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recruited by murine FcγRIIB. Identical results were observed in murine and in human B cells. We demonstrate that SHIP is necessary for human FcγRIIB to inhibit BCR signaling, and cannot be replaced by SHP-1 or SHP-2. Although it contains no tyrosine, the C-terminal segment of human FcγRIIB was as mandatory as the tyrosine-containing C-terminal segment of murine FcγRIIB for SHIP1 to be recruited to the ITIM. This segment, however, did not recruit the adapters Grb2/Grap which were demonstrated to stabilize the recruitment of SHIP1 to the ITIM in murine FcγRIIB.

The SH2 domain-containing inositol 5-phosphatase SHIP1 is recruited to the intracytoplasmic domain of human FcγRIIB and is mandatory for negative regulation of B cell activation.

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Summary

Murine Fc γ RIIB were demonstrated to recruit SH2 domain-containing inositol 5-phosphatases (SHIP1/2), when their ITIM is tyrosyl-phosphorylated upon co-aggregation with BCR, and SHIP1 to account for Fc γ RIIB-dependent negative regulation of murine B cell activation. Although human Fc γ RIIB share the same ITIM as murine Fc γ RIIB and similarly inhibit human B cell activation, which among the four known SH2 domain-containing (tyrosine or inositol) phosphatases is/are recruited by human Fc γ RIIB is unclear. Our recent finding that, besides the ITIM, a second tyrosine-based motif is mandatory for murine Fc γ RIIB to recruit SHIP1 challenged the possibility that human Fc γ RIIB recruit this phosphatase. Human Fc γ RIIB indeed lack this motif. Using an experimental model which enabled us to compare human Fc γ RIIB and murine Fc γ RIIB under strictly controlled conditions, we show that SHIP1 is recruited to the intracytoplasmic domain of human Fc γ RIIB and inhibits the same biological responses and intracellular signals as when recruited by murine Fc γ RIIB. Identical results were observed in murine and in human B cells. We demonstrate that SHIP is necessary for human Fc γ RIIB to inhibit BCR signaling, and cannot be replaced by SHP-1 or SHP-2. Although it contains no tyrosine, the C-terminal segment of human Fc γ RIIB was as mandatory as the tyrosine-containing C-terminal segment of murine Fc γ RIIB for SHIP1 to be recruited to the ITIM. This segment, however, did not recruit the adapters Grb2/Grap which were demonstrated to stabilize the recruitment of SHIP1 to the ITIM in murine Fc γ RIIB.

Introduction

FcγRIIB are low-affinity receptors for the Fc portion of IgG, which were demonstrated to negatively regulate B Cell Receptor (BCR)-dependent B cell activation [1-3]. Confirming *in vivo* the inhibitory properties of FcγRIIB on B cell activation demonstrated *in vitro*, FcγRIIB-deficient mice were found to exhibit enhanced antibody responses [4], to be hyperresponsive to collagen-induced arthritis [5,6], and to develop spontaneous systemic lupus erythematosus when in the C57BL/6 background [7].

To exert their regulatory properties in B cells, FcγRIIB need to be coaggregated with BCR by IgG immune complexes [8]. Coaggregation enables FcγRIIB to be tyrosyl-phosphorylated [9] by the src kinases that are associated with BCR [10]. When phosphorylated, murine FcγRIIB recruit the single SH2 domain-containing inositol 5'-phosphatases SHIP1 [11,12] and SHIP2 [13]. Two tyrosine residues, contained in the intracytoplasmic domain of murine FcγRIIB, were found to be critical for the recruitment of SHIP1 [11,14]. One tyrosine is constitutive of an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) [15,16]. Due to the presence of two leucine residues at position Y+2 and Y+3, this motif specifically binds the SH2 domain of SHIP1 when phosphorylated [17]. It is mandatory for the recruitment of SHIP1 by murine FcγRIIB. We recently found that a second tyrosine-based motif, C-terminal to the ITIM, is also necessary for SHIP1 to be recruited by murine FcγRIIB. This motif binds the cytosolic adapters Grb2 and/or Grap which, by interacting with SHIP1 *via* their C-terminal SH3 domain, form a stable tri-molecular complex involving the receptor, the adapter and the phosphatase [14].

When recruited by FcγRIIB, SHIP1 is brought near the plasma membrane, where it can hydrolyze the 5-phosphate group in phosphatidylinositol-(3,4,5)-trisphosphate [PI(3,4,5)P3]. PI(3,4,5)P3, generated by phosphatidylinositol-3 Kinase following BCR

aggregation [18,19], provides docking sites for Pleckstrin Homology (PH) domain-containing molecules that critically contribute to BCR signaling. These include the Bruton's Tyrosine Kinase (Btk) and Phospholipase C- γ (PLC γ) [20]. When phosphorylated by Btk [21] and by the tyrosine kinase Syk [22], PLC γ is activated and generates inositol-1,4,5-trisphosphate, which triggers the mobilization of intracellular Ca²⁺ stores and, secondarily, an influx of extracellular Ca²⁺. Fc γ RIIB-dependent inhibition of the Ca²⁺ response can therefore be accounted for by the catalytic activity of SHIP1. When recruited by Fc γ RIIB, SHIP1 becomes phosphorylated, and it also functions as an adaptor molecule. It indeed recruits the adapter molecule Dok1, which in turn recruits RasGAP, which enhances the autocatalytic GTPase activity of rasGTP and promotes a shift towards the rasGDP inactive form [23,24]. SHIP1 can therefore also inhibit the ras pathway independently of its phosphatase activity. Taken together, these results indicate that SHIP1 plays a central role in the inhibition of B cell responses by murine Fc γ RIIB. Supporting this conclusion, Fc γ RIIB-dependent inhibition of BCR-induced Ca²⁺ mobilization and Erk activation was markedly reduced in B cells from SHIP1-deficient mice [25,26].

Human Fc γ RIIB were also shown to inhibit both the Ca²⁺ response [27,28] and the ras pathway [3], when coaggregated with BCR in human B cells. Human Fc γ RIIB contain the same ITIM as murine Fc γ RIIB, which is likely to recruit SH2 domain-containing phosphatases when phosphorylated. Four SH2 domain-containing phosphatases have been described: the two protein tyrosine phosphatases SHP-1 and SHP-2, and the two inositol phosphatases, SHIP1 and SHIP2. Which, among these phosphatases, is/are responsible for human Fc γ RIIB-dependent negative regulation is unclear. Koncz *et al.* first reported the coprecipitation of SHP-2, but not that of SHIP1 or SHP-1 with human Fc γ RIIB, when coaggregated with BCR in human B cells [29], whereas Ehrhardt *et al.* reported the

coprecipitation of SHIP1, but not of SHP-1 or SHP-2 with human Fc γ RIIB expressed in murine B cells [30]. These studies were performed in different cells, as well as other studies which suggested a role for either protein tyrosine phosphatases or inositol phosphatases [31-33]. Our recent finding that a second tyrosine-based motif is mandatory for murine Fc γ RIIB to recruit SHIP1 made the possibility that human Fc γ RIIB could recruit this phosphatase unlikely. Although they contain an ITIM, human Fc γ RIIB indeed lack the C-terminal tyrosine-based motif.

To determine whether human Fc γ RIIB use different or the same phosphatases as murine Fc γ RIIB to inhibit B cell activation when coaggregated with BCR, we constructed chimeric receptors in which the intracytoplasmic domain of murine Fc γ RIIB had been replaced by the intracytoplasmic domain of human Fc γ RIIB. Wild-type murine Fc γ RIIB and murine-human chimeras were stably expressed in murine and in human B cells, and the responses of these cells were compared following coaggregation of recombinant receptors with endogenous BCR using the same extracellular ligands in the same cells. We found that the intracytoplasmic domain of human Fc γ RIIB triggers the same inhibitory signals as the intracytoplasmic domain of murine Fc γ RIIB and, surprisingly, that it selectively recruits SHIP1 in both murine and human B cells. The recruitment of SHIP1, however, also required the C-terminal segment of human Fc γ RIIB although no tyrosine is contained in this segment which did not recruit Grb2. Finally, we provide genetic evidence that SHIP1 is necessary for human Fc γ RIIB to negatively regulate B cell activation.

Materials and Methods

Cells. IIA1.6 cells were cultured in RPMI supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.5 µM 2-mercaptoethanol and 2mM sodium pyruvate. ST486 cells, a gift of Dr. G. Sarmay (Eötvös University, Budapest, Hungary) were cultured in OptiMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. DT40 cells, purchased from Riken Cell Bank (Tsukuba Science City, Japan), were cultured in RPMI supplemented with 10% FCS, 1% chicken serum 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.5 µM 2-mercaptoethanol.

Antibodies. The rat anti-mouse FcγRIIB 2.4G2 mAb [34] was purified on protein G-Sepharose. F(ab')₂ fragments and IgG of polyclonal Rat anti-Mouse Ig (R_{at}AM), F(ab')₂ fragments and IgG of polyclonal Rabbit anti-Mouse Ig (R_{ab}AM), and IgG of polyclonal Rabbit anti-Chicken Ig (R_{ab}AC) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), rabbit anti-phospho-Akt and anti-Akt antibodies and rabbit anti-phospho-Erk and anti-Erk antibodies from New England Biolabs (Beverly, MA), mouse anti-Grb2, mouse anti-SHP-1 and mouse anti-SHP-2 antibodies from Transduction Laboratories (Lexington, KY), mouse anti-phosphotyrosine mAbs (4G10), rabbit anti-SHIP1 from Upstate Biotechnology (Lake Placid, NY), HRP-conjugated Goat anti-Rabbit (HRP-GAR) and Goat anti-Mouse (HRP-GAM) Ig antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against recombinant EC domains of FcγRIIB were a gift from Pr. C. Sautès-Fridman (INSERM U255, Paris, France). Rabbit anti-human SHIP1 antibodies were a gift from Dr. S. Latour (INSERM U429, Paris, France). Rabbit anti-SHIP1 antibodies used in

DT40 cells were a gift from Dr. J. V. Ravetch (The Rockefeller University, New York, NY).

cDNA constructs. The cDNA of mouse Fc γ RIIB was modified by a point mutation of the codon coding for V₂₁₂ (GTT->GTA), which induced no amino acid change, but created a KpnI restriction site. This cDNA encoding the extracellular and transmembrane domains and the six first intracytoplasmic amino acids of Fc γ RIIB was inserted into an expression vector under the control of the SR α promoter in pBR322 [35] and in which a neomycin-resistance or a zeocin-resistance gene was introduced. cDNAs encoding the intact or truncated intracellular domain of human, murine or chimeric Fc γ RIIB were amplified using the following primers. B1mh: primer 1 (5'- GCC TTG GTA CCT CTC AGG AAA AAG CGG ATT-3') and primer 2 (5'- ACA ATG GAG CTC AAA TAC GGT TCT GGT CAT-3'). B1mh Δ 297: primer 1 and primer 3 (5'-CTC TTC CAG AGC TCC CTA GTG CAT GAG AAG-3'). B1mm Δ 314 + hC-ter: primer 4 (5'- AAG AAA AAG CAG GTA CCA GCT CTC CCA-3') and primer 5 (5'-CG AGC TCA AAT ACG GTT CTG GTC ATC AGG CTC TTC CAG AGC ATC CGG ATG CTT GAG AAG TGA GTA GGT-3'). These cDNAs were then fused to the cDNA encoding the extracellular and transmembrane domains of Fc γ RIIB. B1mm Δ 314 was already described [36].

Transfectants. cDNAs were stably transfected in IIA1.6, ST486 and DT40 cells by electroporation. Transfectants were selected and cloned as described [37-39]. The expression of recombinant receptors was assessed by indirect immunofluorescence using the rat mAb 2.4G2 and Fluorescein Isothiocyanate-labeled F(ab')₂ fragments of Mouse anti-Rat Ig (FITC-MAR F(ab')₂) (Jackson ImmunoResearch). The expression of receptors on clones remained stable over the duration of experiments. Several clones of each transfectant were used and gave similar results.

IL-2 secretion. Aliquots of 5×10^5 IIA1.6 transfectants, resuspended in culture medium and distributed in 96-well microculture plates, were incubated with various concentrations of IgG or $F(ab')_2 R_{ab}AM$ for 18 h at $37^\circ C$. Cell-free supernatants were harvested and assayed for IL-2 on CTLL-2 cells as previously described [40].

Flow cytometric analysis of Ca^{2+} mobilization. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by preloading 1×10^6 IIA1.6 or DT40 cells with 5 mM Fluo-3 AM (Molecular Probes, Eugene, OR) in the presence of 0.2 % Pluronic F-127 (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. Cells were washed 3 times in RPMI, resuspended at 1×10^6 cells/ml in complete medium, and warmed for 3 min at $37^\circ C$ before they were challenged with equimolar concentrations of intact or $F(ab')_2$ fragments of anti-BCR antibodies. IIA1.6 cells were stimulated with $45 \mu g/ml$ $R_{ab}AM$ IgG or $30 \mu g/ml$ $R_{ab}AM$ $F(ab')_2$. DT40 cells were stimulated with $135 \mu g/ml$ $R_{ab}AC$ IgG or $90 \mu g/ml$ $R_{ab}AC$ $F(ab')_2$. Mean $[Ca^{2+}]_i$ was monitored by flow cytometry using a FACS Calibur and calculated using the software FCS assistant 1.2.9 beta (Becton Dickinson).

In vivo analysis of Akt translocation. The construct encoding the Akt PH domain fused to enhanced Green Fluorescent Protein (GFP) was kindly provided by Dr. Tobias Meyer (Stanford University School of Medicine, Stanford, CA). Cells were transiently transfected with 5 μg of DNA by electroporation (280V, 960 μF) and cultured for 16 h in complete culture medium before analysis. Fluorescence microscopy was performed with a Leitz DM IRB inverted microscope (Leica, Wetzlar, Germany) using a x100 oil objective on viable cells, seeded on glass coverslips placed in 30-mm Petri dishes. Cells were stimulated with $10 \mu g/ml$ $R_{ab}AM$ $F(ab')_2$ or $15 \mu g/ml$ $R_{ab}AM$ IgG, and fluorescence was analyzed after a 10-min incubation at $37^\circ C$. Fluorescence images were collected with a cooled CDD camera

(Micomax, Princeton Scientific Instruments, Inc, Monmouth Junction, NJ) and the Metamorph software (Universal Imaging Corporation, Downingtown, PA). For image processing and presentation, digital images (8 bits scale) were printed directly.

Whole cell lysate analysis. IIA1.6 and ST486 transfectants were stimulated at 37°C for the indicated times with 30 µg/ml intact or 20 µg/ml F(ab')₂ fragments of R_{at}AM IgG or R_{ab}AH IgG, and lysed by 3 cycles of incubation for 1 min in liquid nitrogen followed by 1 min at 37°C in lysis buffer pH 8.0 (50 mM Tris pH8, 150 mM NaCl, 1% Tx100, 1 mM Na₃VO₄, 5 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1mM PMSF). Proteins were quantitated using a Biorad protein assay (Hercules, CA), and 40 µg proteins were treated as described in *Western blot analysis*.

Immunoprecipitation. IIA1.6 and ST486 transfectants were stimulated at 37°C for 3 min with 30 µg/ml R_{ab}AM IgG or R_{ab}AH IgG. Cells were lysed in lysis buffer pH 7.4 (10 mM Tris pH 7.4, 150 mM NaCl, 1% Tx100, 1 mM Na₃VO₄, 5 mM NaF, 5 mM Sodium pyrophosphate, 0.4 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mM PMSF). Postnuclear lysates were immunoprecipitated with 2.4G2-coated sepharose beads. Eluates from immunoadsorbents were treated as described in *Western blot analysis*.

Western blot analysis. Material was boiled in Sample Buffer, fractionated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were saturated with either 5% BSA (Sigma Chemical Co) or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) diluted in Western buffer (150 mM NaCl, 10 mM Tris and 0.5% Tween 20 (Merk, Schuchardt, Germany) pH7.4), and incubated with the indicated antibodies followed by HRP-GAR or HRP-GAM. Labeled antibodies were detected using an ECL kit (Amersham Pharmacia biotech, Little Chalfont, Buckinghamshire).

Results

A chimeric receptor containing the intracytoplasmic domain of human Fc γ RIIB inhibits BCR signaling in murine B cells.

Chimeric receptors, containing the extracellular and transmembrane domains of murine Fc γ RIIB and the intracytoplasmic domain of human Fc γ RIIB1 (B1mh) were constructed and stably expressed in the Fc γ R-deficient variant of the murine B lymphoma A20/2J, IIA1.6. Wild-type (B1mm) and intracytoplasmic domain-deleted (B1m) murine Fc γ RIIB1 expressed in the same cells were used as positive and negative controls, respectively (Fig. 1). BCR were either aggregated using rabbit anti-mouse Ig (R_{ab}AM) F(ab')₂ fragments, or coaggregated with Fc γ RIIB using intact R_{ab}AM IgG antibodies, and Fc γ RIIB-dependent inhibition was assessed by comparing biological responses and intracellular signals elicited in IIA1.6 cells under these two conditions.

B1mh abolished BCR-dependent IL-2 secretion as efficiently as B1mm, but not B1m (Fig. 2A). B1mh inhibited the late phase of the BCR-dependent Ca²⁺ response as efficiently as B1mm, but not B1m (Fig. 2B). B1mh inhibited BCR-dependent phosphorylation of the MAP Kinases Erk1/2 as efficiently as B1mm, but not B1m (Fig. 2C). B1mh, B1mm or B1m, however, did not detectably decrease BCR-dependent tyrosyl-phosphorylation of proteins in whole cell lysates (not shown).

We next examined signaling events that depend on the generation of membrane phospholipids. Akt is phosphorylated by PDK1 [41] when both molecules are translocated to the membrane *via* the interaction of their PH domain with PI(3,4,5)P3 or PI(4,5)P2. Akt phosphorylation was induced with similar intensities in the three transfectants upon BCR aggregation. It was inhibited when BCR were coaggregated with B1mh and B1mm, but not

with B1m (Fig. 2D). To assess more directly membrane PI(3,4,5)P3, a GFP construct containing the PH domain of Akt was transiently expressed in the three transfectants. GFP had a diffuse cytosolic distribution in unstimulated cells. It was translocated to the membrane upon BCR aggregation. This translocation was prevented when BCR were coaggregated with either B1mh or B1mm, but not with B1m (Fig. 2E).

These results altogether indicate that chimeric receptors containing the intracytoplasmic domain of human FcγRIIB1 can inhibit BCR-dependent cytokine secretion, Ca²⁺ response, ras pathway activation, membrane translocation and phosphorylation of Akt as efficiently as murine FcγRIIB1, without affecting the overall intracellular protein phosphorylation.

SHIP1 is recruited to the intracytoplasmic domain of human FcγRIIB1 in murine and in human B cells.

To identify phosphatases possibly recruited by human FcγRIIB, B1mh and B1mm were coaggregated with BCR by R_{ab}AM IgG antibodies in IIA1.6 transfectants, and immunoprecipitated using the same anti-murine FcγRIIB mAb. B1mh was tyrosyl-phosphorylated when coaggregated with BCR, albeit to a lower extent than B1mm. SHIP1, but neither SHP-1 nor SHP-2, coprecipitated with B1mh and, as expected, with B1mm. Noticeably, comparable amounts of SHIP1 coprecipitated with B1mm and B1mh, in spite of the lower phosphorylation of the latter (Fig. 3A).

To exclude that B1mh behaved like B1mm because they were expressed in murine cells, the same two constructs were stably expressed in ST486, a human B lymphoma cell line that lacks endogenous FcγR. ST486 clones expressing comparable amounts of recombinant receptors were selected (Fig. 1). B1mh and B1mm were coaggregated with BCR using rabbit

anti-human Ig (R_{ab}AH) IgG antibodies. Under these conditions, B1mh was, again, less phosphorylated than B1mm, and SHIP1, but neither SHP-1 nor SHP-2, coprecipitated with both B1mh and B1mm in ST486 cells (Fig. 3B).

A chimeric receptor containing the intracytoplasmic domain of human FcγRIIB1 therefore recruited SHIP1 selectively, when tyrosyl-phosphorylated upon coaggregation with BCR, in both murine and human B cells.

The recruitment of SHIP1 requires the 13 C-terminal aminoacids in the intracytoplasmic domain of human FcγRIIB.

We recently demonstrated that, in addition to the ITIM, a second tyrosine-based motif is required for murine FcγRIIB1 to recruit SHIP1 [14]. This second motif, C-terminal to the ITIM, is conserved in all three isoforms of murine FcγRIIB, but not in the two isoforms of human FcγRIIB. We investigated whether, although it contains no tyrosine, the C-terminal segment of the intracytoplasmic domain of human FcγRIIB, could play the same role as the tyrosine-containing C-terminal segment of the intracytoplasmic domain of murine FcγRIIB.

B1mh chimeras deleted of their 13 C-terminal intracytoplasmic aminoacids (B1mh Δ297) were constructed and expressed in IIA1.6 cells. These receptors were compared with murine FcγRIIB1 deleted of the 16 C-terminal intracytoplasmic aminoacids (B1mm Δ314) expressed in the same cells (Fig. 1). Non-deleted B1mh and B1mm served as positive controls. As previously observed [36], SHIP1 failed to coprecipitate with B1mm Δ314, which was phosphorylated upon coaggregation with BCR. SHIP1 also failed to coprecipitate with B1mh Δ297, which was tyrosyl-phosphorylated under the same conditions (Fig. 4). Although they contain no tyrosine, the 13 C-terminal aminoacids of the intracytoplasmic domain of human FcγRIIB are therefore necessary for these receptors to recruit SHIP1.

To investigate their role in the recruitment of SHIP1, the 13 C-terminal aminoacids of human FcγRIIB were used to replace the tyrosine-containing C-terminal 16 aminoacids that were deleted in B1mm Δ314. The resulting chimera (B1mm Δ314+hCter) was expressed in IIA1.6 cells (Fig. 1) and coaggregated with BCR. SHIP1 coprecipitated with tyrosyl-phosphorylated B1mm Δ314+hCter (Fig. 4). The 13 C-terminal aminoacids of human FcγRIIB could therefore restore the ability of B1mm Δ314 to recruit SHIP1. B1mh, however, lacks the C-terminal tyrosine-based motif which we demonstrated to recruit Grb2 [14], and, as expected, Grb2 failed to coprecipitate with B1mh, when the latter was coaggregated with BCR in IIA1.6 cells (Fig. 4). Supporting this observation, a 13-aminoacid phosphorylated synthetic peptide corresponding to the 13 C-terminal aminoacids of human FcγRIIB failed to bind Grb2 when incubated in IIA1.6 cell lysates (data not shown). It also failed to bind SHIP1.

Altogether, these results indicate that, like the intracytoplasmic domain of murine FcγRIIB, the intracytoplasmic domain of human FcγRIIB requires a C-terminal sequence, in addition to the ITIM, to recruit SHIP1. The mechanism by which their C-terminal sequence enables human FcγRIIB to recruit SHIP1 is however different from the mechanism by which their C-terminal sequence enables murine FcγRIIB to recruit SHIP1.

SHIP1 is mandatory for inhibition of BCR-dependent B cell activation by human FcγRIIB1.

SHIP1 being selectively coprecipitated with B1mh, we investigated whether this phosphatase is necessary for human FcγRIIB to inhibit BCR-dependent B cell activation. Since both SHIP1 and SHP-2 were reported to coprecipitate with human FcγRIIB [29,30], B1mh and B1mm were expressed in wild-type, SHIP-deficient, or SHP-2-deficient DT40

chicken B cells. BCR aggregation, induced by F(ab')₂ fragments of rabbit anti-chicken Ig (R_{ab}AC) antibodies, increased the intracellular Ca²⁺ concentration in all three DT40 transfectants. As expected, the Ca²⁺ response was shortened when BCR were coaggregated with either B1mh or B1mm by intact R_{ab}AC IgG in wild-type DT40 cells. B1mm-induced inhibition of the Ca²⁺ response was abrogated in SHIP-deficient cells, as previously described, but not in SHP-2-deficient cells. B1mh-induced inhibition of the Ca²⁺ response was also abrogated in SHIP-deficient cells, but not in SHP-2-deficient cells (Fig. 5). SHIP1 is therefore necessary not only for murine FcγRIIB-, but also for human FcγRIIB-dependent inhibition of BCR signaling.

Discussion

We document here the molecular mechanism by which human FcγRIIB inhibit BCR signaling. We found that the SH2 domain-containing inositol 5-phosphatase SHIP1 is recruited to the intracytoplasmic domain of human FcγRIIB and inhibits the same biological responses and intracellular signals as when recruited by murine FcγRIIB. We show that SHIP is necessary not only for murine, but also for human FcγRIIB to inhibit BCR signaling, and cannot be replaced by SHP-1 or SHP-2. The mechanism that enables human and murine FcγRIIB to recruit SHIP1 is however different.

The first ITIM was identified as a highly conserved tyrosine-containing sequence, when comparing the intracytoplasmic domains of murine and human FcγRIIB which had been found to exert comparable inhibitory properties on cell activation by ITAM-containing receptors [42]. Phosphorylated peptides corresponding to this sequence were found to bind *in vitro* the four known SH2 domain-containing phosphatases when immobilized on beads and incubated in murine [15] or in human [29,31] cell lysates. Although initially reported to

recruit SHP-1 [15], murine Fc γ RIIB was convincingly shown to selectively recruit SHIP1 when co-engaged with BCR in B cells or with Fc ϵ RI in mast cells [11,12,43,44]. Discordant results were reported by several groups who investigated the recruitment of phosphatases by human Fc γ RIIB. Several reasons may explain these inconsistencies. No mAb specific for human Fc γ RIIB is available, and human B cells express not only Fc γ RIIB, but also ITAM-containing Fc γ RIIA. The extracellular domains of the two receptors being highly conserved, both Fc γ RIIB and Fc γ RIIA are co-aggregated with BCR when engaged by IgG immune complexes or by intact IgG anti-human Ig on human B cells. Available mAbs also precipitate less efficiently human Fc γ RIIB, compared to murine Fc γ RIIB. In addition, human Fc γ RIIB is less tyrosyl-phosphorylated than murine Fc γ RIIB, when coaggregated with BCR. The intracytoplasmic domain of human Fc γ RIIB1 indeed contains two tyrosines only, whereas that of murine Fc γ RIIB1 contains four tyrosines. Finally, different cell types and different experimental conditions were used by different groups who did not always distinguish the *in vitro* binding of phosphatases to phospho-peptides, the *in vivo* coprecipitation of phosphatases with Fc γ RIIB in pervanadate-treated cells, and the *in vivo* coprecipitation of phosphatases with Fc γ RIIB when co-aggregated with BCR. In order to overcome these difficulties, we generated an experimental model which enabled us to compare human Fc γ RIIB and murine Fc γ RIIB under strictly controlled conditions. Clones of transfectants expressing comparable levels of murine Fc γ RIIB1 (B1mm), murine-human chimeric Fc γ RIIB1 (B1mh) or tail-less murine Fc γ RIIB (B1m) could be selected using the same mAb to assess receptor expression. B1mm and B1mh could be both expressed and compared in the same cells *i.e.* in murine B cells, in human B cells or in chicken B cells which otherwise express no other Fc γ R. The same extracellular ligands could be used to co-aggregate BCR with B1mm and B1mh, and the

same biological responses could be examined in each B cell type. Finally, the same antibodies could be used to precipitate B1mm and B1mh with the same efficiency in all B cells.

Using this experimental model, we found that SHIP1 is the effector of human Fc γ RIIB-dependent negative regulation of B cell activation. First of all, B1mm and B1mh comparably inhibited BCR-dependent IL-2 secretion and major signaling events, including MAP kinase activation, Ca²⁺ mobilization, Akt phosphorylation and PI(3,4,5)P3 generation, as assessed by the translocation of a GFP-PH_{Akt} construct, in murine B cells. Second, SHIP1 similarly coprecipitated with B1mm and B1mh, when tyrosyl-phosphorylated upon co-aggregation with BCR. Identical results were observed in IIA1.6 and in ST486 cells, excluding that murine and human Fc γ RIIB could function differently in murine and human B cells, respectively. Our results are consistent with previous papers reporting that SHIP1 was recruited by human Fc γ RIIB in IIA1.6 cells [30], and that inhibition of Fc γ RIIA-mediated phagocytosis observed upon coaggregation with human Fc γ RIIB was enhanced in COS cells overexpressing SHIP1 [33]. Noticeably, neither SHP-1 nor SHP-2 coprecipitated with B1mh when coaggregated with BCR in IIA1.6 or in ST486 cells. SHP-2 was previously described to coprecipitate with human Fc γ RIIB in a human B cell line [29] and in melanoma cells treated with pervanadate [45]. Likewise, SHP-1 coprecipitated with murine Fc γ RIIB in pervanadate-treated B cells, but not following coaggregation with BCR [44]. Finally, the deletion of SHIP abrogated the inhibition of Ca²⁺ mobilization by both B1mm and B1mh in DT40 cells. SHIP, but not SHP-2, is therefore used by and mandatory for human Fc γ RIIB to inhibit BCR signaling. Supporting this conclusion, no major alteration of the pattern of tyrosyl-phosphorylation was observed in whole cell lysates following BCR aggregation or following the co-aggregation of BCR with B1mm or B1mh in IIA1.6 cells.

Our experimental model also enabled us to show that, like for murine Fc γ RIIB, the

ITIM is not sufficient for enabling human FcγRIIB to recruit SHIP1. Their C-terminal segment is indeed necessary for both receptors to recruit the phosphatase. Different mechanisms, however, stabilize the binding of SHIP1 to the ITIM in human and murine FcγRIIB. We have recently shown that SHIP1 needs the cooperation of the cytosolic adapters Grb2 and/or Grap in order to be recruited by murine FcγRIIB. These adapters are recruited to a second tyrosine-containing motif, located C-terminal to the ITIM in the intracytoplasmic domain of FcγRIIB [14]. This finding was puzzling as human FcγRIIB contain the same ITIM as murine FcγRIIB, but they lack this second motif in their C-terminal segment. Another tyrosine, located N-terminal to the ITIM, in human FcγRIIB1 could have possibly played the same role, in human FcγRIIB, as that played by the C-terminal motif in murine FcγRIIB. A point mutation of this tyrosine, however, did not impair the ability of human FcγRIIB1 to inhibit BCR-induced Ca²⁺ mobilization [46]. Surprisingly, the deletion of their 13 C-terminal aminoacids made B1mh unable to detectably coprecipitate SHIP1. The contribution of these aminoacids to the recruitment of SHIP1 was formally demonstrated by replacing the tyrosine-containing C-terminal segment of murine FcγRIIB by the C-terminal segment of human FcγRIIB. This substitution indeed restored the ability of the murine FcγRIIB deletant to recruit SHIP1, but not Grb2. Although it contains no tyrosine, the C-terminal segment of human FcγRIIB therefore plays the same role as the C-terminal segment of murine FcγRIIB. How it stabilizes the recruitment of SHIP1 is unknown. As a synthetic peptide corresponding to this segment failed to bind SHIP1 *in vitro*, the simplest possibility is that it recruits another molecule that can also bind to SHIP1. We however failed to coprecipitate any of the previously described binding partners of SHIP1 with B1mh, and no additional band was observed in silver-stained B1mh immunoprecipitates, when compared with B1mm immunoprecipitates in IIA1.6 cells (not shown). If one aligns residues in the C-terminal

segments of murine and human Fc γ RIIB, one observes that 10 of 13 aminoacids are identical or similar. Putative molecules that could interact both with this sequence in human Fc γ RIIB and with SHIP1 remain to be identified.

In conclusion, this work demonstrates that SHIP1 is the effector of the inhibition of B cell activation by human Fc γ RIIB, when recruited to the phosphorylated ITIM and stabilized by the C-terminal segment through an unknown molecular interaction. Interestingly, the expression of SHIP1 is highly regulated in hematopoietic cells. Thus, SHIP1 expression was reported to be high in immature B cells and to decrease as cell differentiate into mature B cells [47]. Fc γ RIIB-dependent negative regulation of cell activation may therefore vary as human B cells mature and differentiate. Whether abnormally high or low levels of SHIP1 are associated with pathological conditions involving defects in Fc γ RIIB-dependent negative regulation is an interesting possibility to investigate.

References

- [1] Daëron M. Fc Receptor Biology. *Annu Rev Immunol* 1997; 15: 203-234.
- [2] Amigorena S, Bonnerot C, Drake J, Choquet D, Hunziker W, Guillet JG, et al. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B-lymphocytes. *Science* 1992; 256: 1808-1812.
- [3] Sarmay G, Koncz G, Gergely J. Human type II Fc γ receptors inhibit B cell activation by interacting with the p21ras-dependent pathway. *J Biol Chem* 1996; 271: 30499-30504.
- [4] Wernersson S, Karlsson MC, Dahlstrom J, Mattsson R, Verbeek JS, Heyman B. IgG-mediated enhancement of antibody responses is low in Fc Receptor γ chain-deficient mice and increased in Fc γ RII-deficient mice. *J Immunol* 1999; 163: 618-22.
- [5] Yuasa T, Kubo S, Yoshino T, Ujike A, Matsumura K, Ono M, et al. Deletion of Fc γ Receptor IIB Renders H-2^b Mice susceptible to Collagen-induced Arthritis. *J Exp Med* 1999; 189: 187-194.
- [6] Nakamura A, Yuasa T, Ujike A, Ono M, Nukiwa T, Ravetch JV, et al. Fc γ Receptor IIB-deficient Mice Develop Goodpasture's Syndrome upon Immunization with Type IV Collagen: A Novel Murine Model for Autoimmune Glomerular Basement Membrane Disease. *J Exp Med* 2000; 191: 899-905.
- [7] Bolland S, Ravetch JV. Spontaneous autoimmune disease in Fc γ RIIB-deficient mice results from strain-specific epistasis. *Immunity* 2000; 13: 277-285.
- [8] Sinclair NRSC, Chan PL. Regulation of the immune responses. IV. The role of the Fc-fragment in feedback inhibition by antibody. *Adv Exp Med Biol* 1971; 12: 609-615.
- [9] Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV. A 13-amino-acid motif in the cytoplasmic domain of Fc γ RIIB modulates B-cell receptor signalling. *Nature* 1994; 368: 70-73.
- [10] Chan VWF, Meng F, Soriano P, DeFranco AL, Lowell CA. Characterization of the B lymphocyte populations in lyn-deficient mice and the role of lyn in signal initiation and down-regulation. *Immunity* 1997; 7: 69-81.
- [11] Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc γ RIIB. *Nature* 1996; 383: 263-266.
- [12] Fong DC, Malbec O, Arock M, Cambier JC, Fridman WH, Daëron M. Selective in vivo recruitment of the phosphatidylinositol phosphatase SHIP by phosphorylated Fc γ RIIB during negative regulation of IgE-dependent mouse mast cell activation. *Immunol Lett* 1996; 54: 83-91.
- [13] Muraille E, Bruhns P, Pesesse X, Daëron M, Erneux C. The SH2 domain containing inositol 5-phosphatase SHIP2 associates to the immunoreceptor tyrosine-based inhibition motif of Fc γ RIIB in B cells under negative signalling. *Immunol Letters* 2000; 72: 7-15.
- [14] Isnardi I, Lesourne R, Bruhns P, Fridman WH, Cambier JC, Daëron M. Two distinct tyrosine-based motifs enable the inhibitory receptor Fc γ RIIB to cooperatively recruit the inositol phosphatases SHIP1/2 and the adapters Grb2/Grap. *J Biol Chem* 2004; 279: 51931-51938.
- [15] D'Ambrosio D, Hippen KH, Minskoff SA, Mellman I, Pani G, Siminovitch KA, et al. Recruitment and activation of PTP1C in negative regulation of antigen receptor

- signaling by Fc γ RIIB1. *Science* 1995; 268: 293-296.
- [16] Vivier E, Daëron M. Immunoreceptor tyrosine-based inhibition motifs. *Immunol Today* 1997; 18: 286-291.
- [17] Bruhns P, Vély F, Malbec O, Fridman WH, Vivier E, Daëron M. Molecular basis of the recruitment of the SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2 by Fc γ RIIB. *J Biol Chem* 2000; 275: 37357-37364.
- [18] Scharenberg AM, El-Hillal O, Fruman DA, Beitz LO, Li Z, Lin S, et al. Phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J* 1998; 17: 1961-1972.
- [19] Bolland S, Pearce RN, Kurosaki T, Ravetch JV. SHIP modulates immune receptor responses by regulating membrane association of Btk. *Immunity* 1998; 8: 509-516.
- [20] Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J. Activation of phospholipase C γ by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO J* 1998; 17: 414-22.
- [21] Takata M, Kurosaki T. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C- γ 2. *J Exp Med* 1996; 184: 31-40.
- [22] Law CL, Chandran KA, Sidorenko SP, Clark EA. Phospholipase C- γ 1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Mol Cell Biol* 1996; 16: 1305-15.
- [23] Tamir I, Stolpa JC, Helgason CD, Nakamura K, Bruhns P, Daëron M, et al. The RasGAP-binding protein p62dok is a Mediator of Inhibitory Fc γ RIIB Signals in B cells. *Immunity* 2000; 12: 347-358.
- [24] Yamanashi Y, Tamura T, Kanamori T, Yamane H, Nariuchi H, Yamamoto T, et al. Role of the rasGAP-associated docking protein p62(Dok) in negative regulation of B cell receptor-mediated signaling. *Gene Develop* 2000; 14: 11-16.
- [25] Liu Q, Oliveira-Dos-Santos AJ, Mariathasan S, Bouchard D, Jones J, Sarao R, et al. The inositol polyphosphate 5-phosphatase ship is a crucial negative regulator of B cell antigen receptor signaling. *J Exp Med* 1998; 188: 1333-42.
- [26] Helgason CD, Damen JE, Rosten P, Grewal R, Sorensen P, Chappel SM, et al. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and shortened life span. *Genes Dev* 1998; 12: 1610-1620.
- [27] Koncz G, Gergely J, Sarmay G. Fc γ RIIB inhibits both B cell receptor- and CD19-induced Ca²⁺ mobilization in Fc γ R-transfected human B cells. *Int Immunol* 1998; 10: 141-146.
- [28] Van den Herik-Oudijk IE, Westerdaal NAC, Henriquez NV, Capel PJA, Van de Winkel JGJ. Functional analysis of human Fc γ RII (CD32) isoforms expressed in B lymphocytes. *J Immunol* 1994; 152: 574-585.
- [29] Koncz G, Pecht I, Gergely J, Sarmay G. Fc γ receptor-mediated inhibition of human B cell activation: the role of SHP-2 phosphatase. *Eur J Immunol* 1999; 29: 1980-1989.
- [30] Ehrhardt GRA, Davis RS, Hsu JT, Leu CM, Ehrhardt A, Cooper MD. The inhibitory potential of Fc receptor homolog 4 on memory B cells. *Proc Natl Acad Sci USA* 2003; 100: 13489-13494.
- [31] Kepley CL, Cambier JC, Morel PA, Lujan D, Ortega E, Wilson BS, et al. Negative regulation of Fc ϵ RI signaling by Fc γ RII costimulation in human blood basophils. *J Allergy Clin Immunol* 2000; 106: 337-348.
- [32] Tridandapani S, Siefker K, Teillaud JL, Carter E, Wewers MD, Anderson CL. Regulated expression and inhibitory function of Fc γ RIIB in human monocytic cells. *J*

- Biol Chem 2002; 277: 5082-5089.
- [33] Huang ZY, Hunter S, Kim MK, Indik ZK, Schreiber AD. The effect of phosphatases SHP-1 and SHIP1 on signaling by the ITIM- and ITAM-containing Fc γ receptors Fc γ RIIB and Fc γ RIIA. *J Leuk Biol* 2003; 73: 823-829.
- [34] Unkeless JC. Characterization of monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. *J Exp Med* 1979; 150: 580-596.
- [35] Takebe Y, Seiki M, Fujisawa JI, Hoy P, Yokota K, Arai KI, et al. SR α promoter: an efficient and versatile mammalian cDNA expression system composed of the Simian Virus 40 early promoter and the R-U5 segment of Human T-cell Leukemia Virus type 1 long terminal repeat. *Mol Cell Biol* 1988; 8: 466-472.
- [36] Fong DC, Brauweiler A, Minskoff SA, Bruhns P, Tamir I, Mellman I, et al. Mutational analysis reveals multiple distinct sites within Fc γ Receptor IIB that function in inhibitory signaling. *J Immunol* 2000; 165: 4453-4462.
- [37] Bruhns P, Marchetti P, Fridman WH, Vivier E, Daëron M. Differential roles of N- and C-terminal ITIMs during inhibition of cell activation by killer cell inhibitory receptors. *J Immunol* 1999; 162: 3168-3175.
- [38] Daëron M, Bonnerot C, Latour S, Fridman WH. Murine recombinant Fc γ RIII, but not Fc γ RII, trigger serotonin release in rat basophilic leukemia cells. *J Immunol* 1992; 149: 1365-1373.
- [39] Takata M, Sabe H, Hata A, Inazu T, Homma Y, Nukada T, et al. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca $^{2+}$ mobilization through distinct pathways. *Embo J* 1994; 13: 1341-1349.
- [40] Gillis S, Smith K. Long-term culture of tumour-specific cytotoxic T-cells. *Nature* 1977; 268: 154-155.
- [41] Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, et al. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 1997; 277: 567-70.
- [42] Daëron M, Latour S, Malbec O, Espinosa E, Pina P, Pasmans S, et al. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc γ RIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity* 1995; 3: 635-646.
- [43] Ono M, Okada H, Bolland S, Yanagi S, Kurosaki T, Ravetch JV. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. *Cell* 1997; 90: 293-301.
- [44] Lesourne R, Bruhns P, Fridman WH, Daëron M. Insufficient Phosphorylation prevents Fc γ RIIB from recruiting the SH2 Domain-Containing Protein Tyrosine Phosphatase SHP-1. *J Biol Chem* 2001: 6327-6336.
- [45] Cassard L, Cohen-Solal J, Galinha A, Sastre-Garau X, Mathiot C, Galon J, et al. Modulation of tumor growth by inhibitory Fc γ receptor expressed by human melanoma cells. *J Clin Invest* 2002; 110: 1549-1557.
- [46] Budde P, Bewarder N, Weinrich V, Schulzeck O, Frey J. Tyrosine-containing sequence motifs of the human immunoglobulin G receptors FcRIIb1 and FcRIIb2 essential for endocytosis and regulation of calcium flux in B cells. *J Biol Chem* 1994; 269: 30636-44.
- [47] Geier SJ, Algate PA, Carlberg K, Flowers D, Friedman C, Trask B, et al. The Human SHIP Gene Is Differentially Expressed in Cell Lineages of the Bone Marrow and Blood. *Blood* 1997; 89: 1876-1885.

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Figure Legends:

Figure 1. Recombinant receptors and chimeras used in this study. Receptors and receptor segments of murine (m) and human (h) origin are represented in white and black, respectively. The ITIM, conserved in murine and human FcγRIIB, is shown as a gray box. The expression of recombinant molecules in IIA1.6 and in ST486 cells was assessed by indirect immunofluorescence using the rat mAb anti-murine FcγRIIB extracellular domain 2.4G2 and FITC-MAR F(ab')₂. Thin histograms, FITC-MAR F(ab')₂ only; thick histograms, 2.4G2 followed by FITC-MAR F(ab')₂.

Figure 2. B1mh inhibits IL-2 secretion, Ca²⁺ mobilization, Erk phosphorylation, Akt phosphorylation and Akt membrane translocation in IIA1.6 cells. (A) *Inhibition of BCR-dependent IL-2 secretion.* IIA1.6 transfectants were stimulated with indicated concentrations of RabAM F(ab')₂ (open circles) or RabAM IgG (closed circles). IL-2 released in 18-h culture supernatants was assayed for [³H]thymidine incorporation in CTLL-2 cells. The figure represents the radioactivity incorporated in CTLL-2 cells as a function of the concentration of RabAM F(ab')₂ or IgG. (B) *Inhibition of BCR-dependent Ca²⁺ mobilization.* IIA1.6 transfectants were loaded with Fluo-3, stimulated with either RabAM F(ab')₂ (grey line) or RabAM IgG (black line) at the time indicated by the arrow, and intracellular Ca²⁺ concentration was monitored by flow cytometry. (C) *Inhibition of BCR dependent Erk phosphorylation.* IIA1.6 transfectants were unstimulated (-) or stimulated (+) with either RatAM F(ab')₂ or RatAM IgG for the indicated periods of time. Cells were lysed, and proteins were fractionated by SDS-PAGE and Western blotted with anti-pErk or anti-Erk antibodies. (D) *Inhibition of BCR-dependent Akt phosphorylation.* IIA1.6 transfectants were unstimulated

(-) or stimulated (+) with either RatAM F(ab')₂ or RatAM IgG for the indicated periods of time. Cells were lysed, and proteins were fractionated by SDS-PAGE and Western blotted with anti-pAkt or anti-Akt antibodies. **(E) Inhibition of BCR-dependent Akt translocation.** IIA1.6 transfectants were transiently transfected with a cDNA construct encoding a fusion protein composed of the Akt PH domain linked to GFP. Cells were unstimulated (medium) or stimulated with either RabAM F(ab')₂ or RabAM IgG for 10 min.

Figure 3. B1mh recruits SHIP1 in murine and in human B cells. **(A) Coprecipitation of phosphatases in IIA1.6 cells.** IIA1.6 transfectants were stimulated (+) or not (-) with RabAM IgG for 3 min. Cells were lysed and FcγRIIB were precipitated. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr, anti-SHIP1, anti-SHP-1 and anti-SHP-2 antibodies. **(B) Coprecipitation of phosphatases in ST486 cells.** ST486 transfectants were stimulated (+) or not (-) with RabAH IgG for 3 min. Cells were lysed and FcγRIIB were precipitated. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr, anti-SHIP1, anti-SHP-1 and anti-SHP-2 antibodies.

Figure 4. The C-terminal segment of the intracytoplasmic domain of human FcγRIIB is necessary for SHIP1 to coprecipitate with B1mh. IIA1.6 transfectants were stimulated (+) or not (-) with RabAM IgG for 3 min. Cells were lysed and FcγRIIB were precipitated. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr, anti-SHIP1 and anti-Grb2 antibodies.

Figure 5. SHIP is mandatory for B1mh-dependent inhibition of Ca²⁺ mobilization in

DT40 cells. B1mm and B1mh were expressed in wt, SHIP^{-/-} and SHP-2^{-/-} DT40 cells. The expression of recombinant receptors in the three types of DT40 cells was assessed by indirect immunofluorescence using 2.4G2 and FITC-MAR F(ab')₂. Thin histograms, FITC-MAR F(ab')₂ only; thick histograms, 2.4G2 and FITC-MAR F(ab')₂. W.t., SHIP^{-/-} and SHP-2^{-/-} DT40 transfectants were loaded with Fluo-3, stimulated with either RabAC F(ab')₂ (grey line) or RabAC IgG (black line) at the time indicated by the arrow, and intracellular Ca²⁺ concentration was measured.

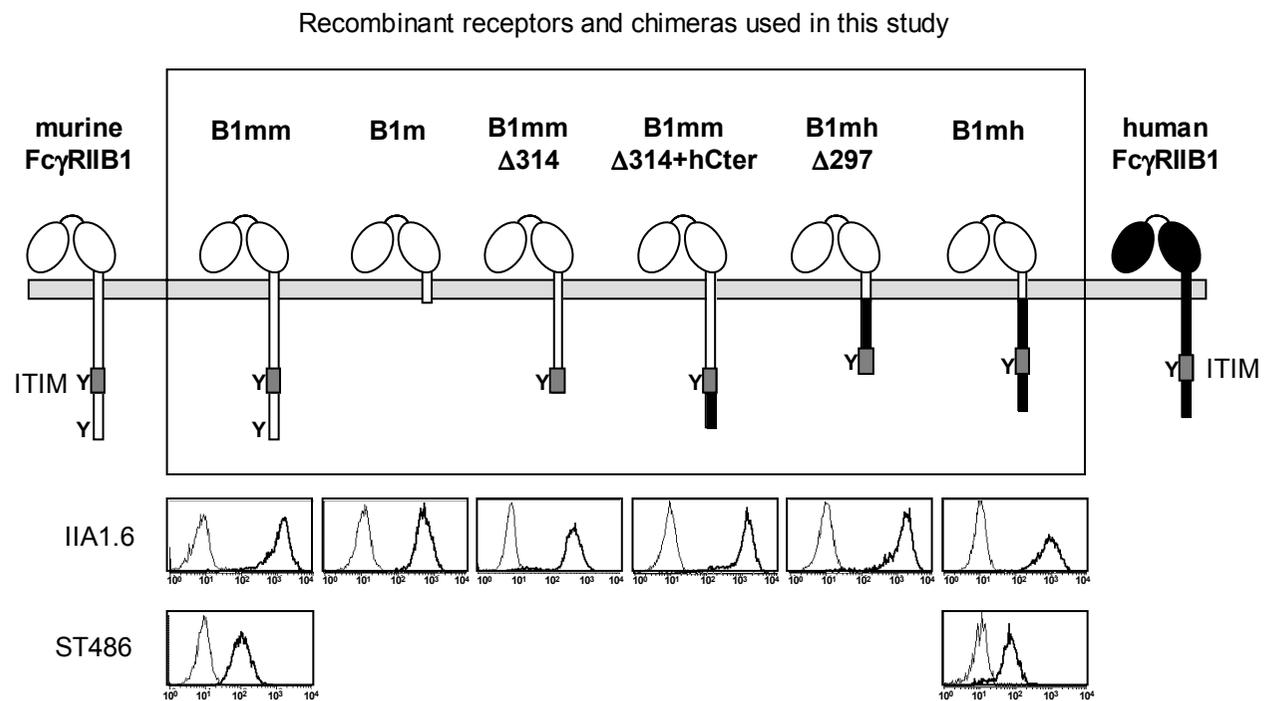


Fig. 1

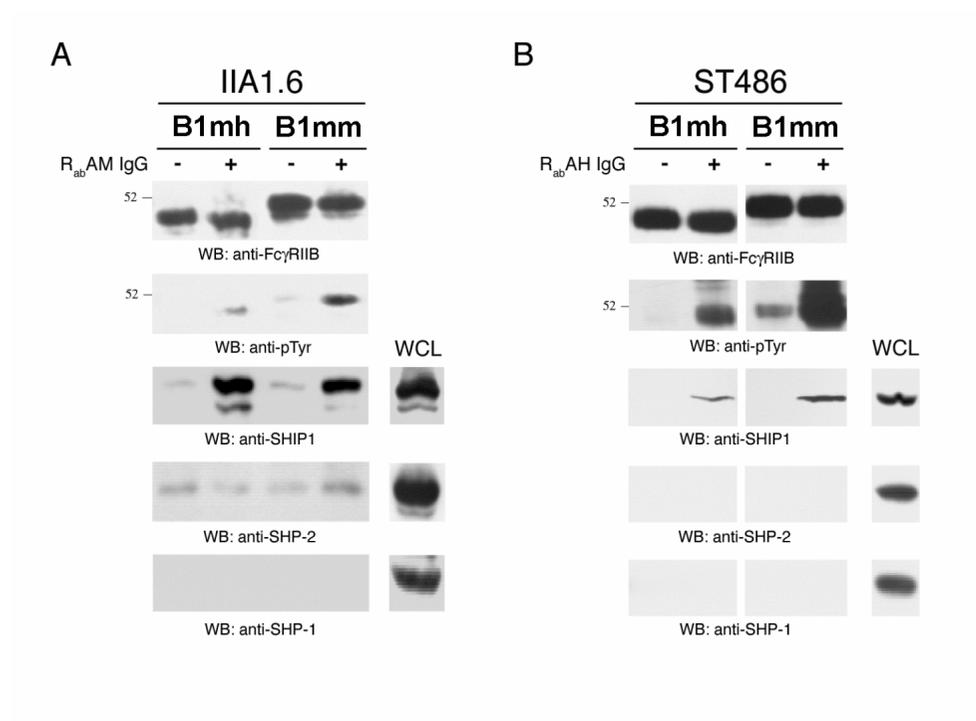


Fig. 3

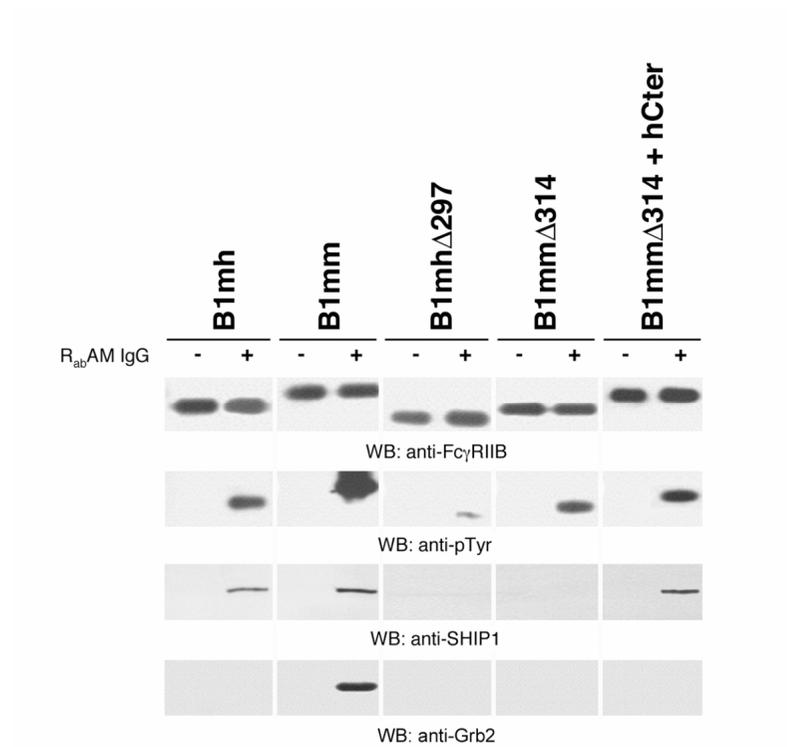


Fig. 4

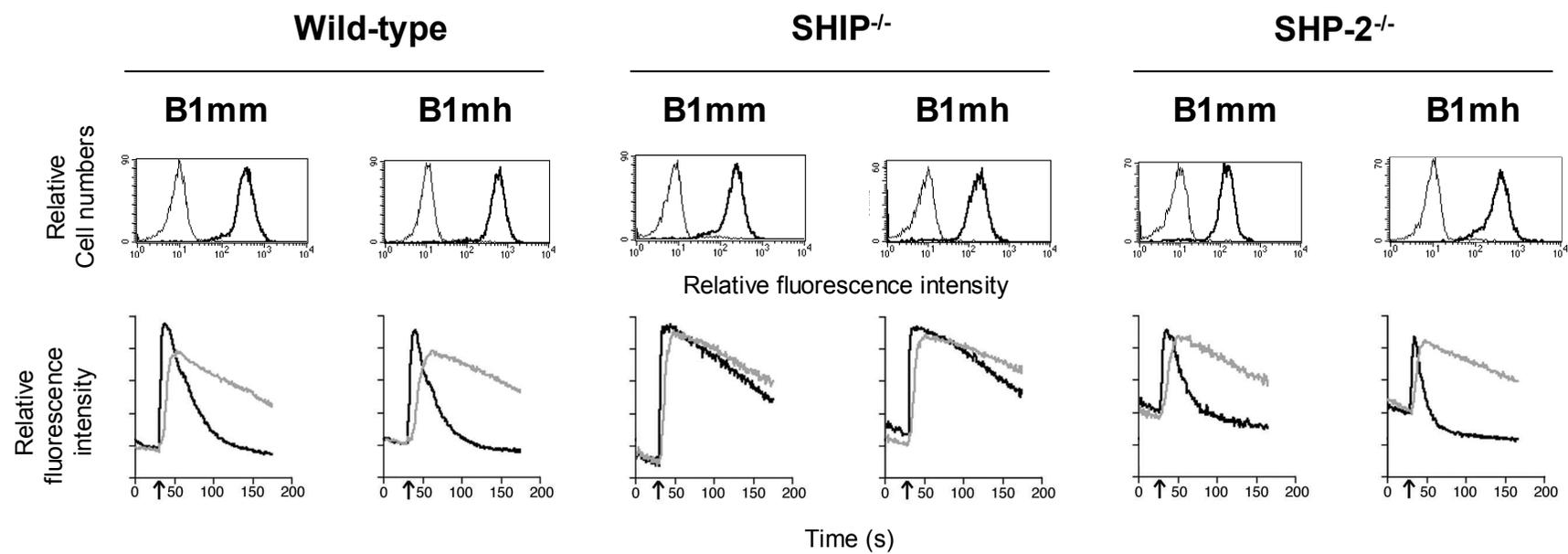
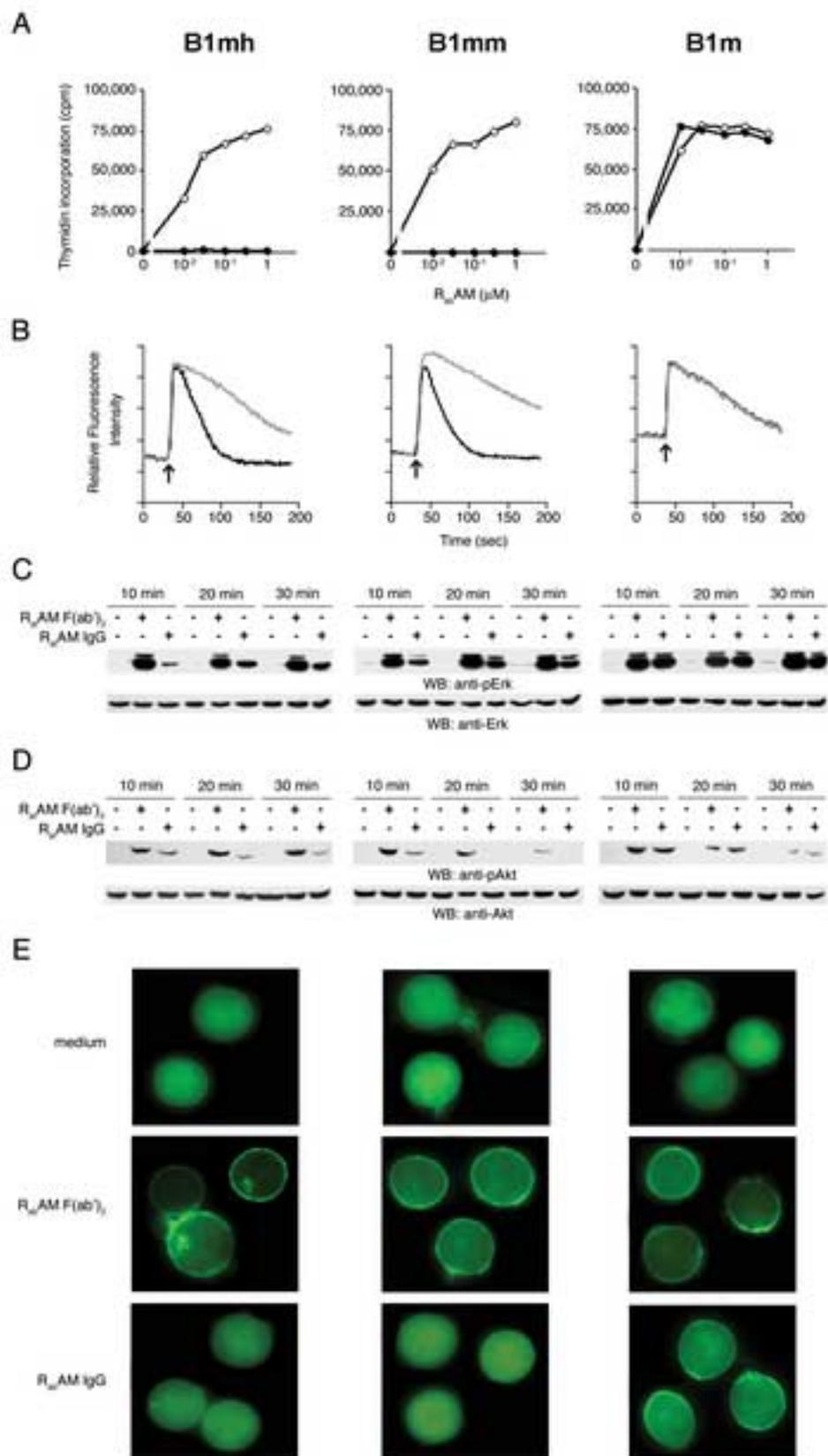


Fig. 5





INSTITUT PASTEUR

Département d'Immunologie
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Paris, November 21, 2005

Dear Vaclav,

The electronic system requests a cover letter. Here it is.

The quality of the pdf version of Fig. 2 being very poor, I have attached a better version.

Please, use it.

Thanks for taking care of our manuscript.

Best regards,

Marc

Marc Daëron, MD, PhD,
Directeur de Recherche à l'INSERM
Chef d'Unité à l'Institut Pasteur

Response to reviewer

Required changes have been made.